

Involvement of Prostaglandin E Receptor Subtype EP₄ in Colon Carcinogenesis¹

Michihiro Mutoh,² Kouji Watanabe,² Tomohiro Kitamura, Yutaka Shoji, Mami Takahashi, Toshihiko Kawamori, Kousuke Tani, Michiyoshi Kobayashi, Takayuki Maruyama, Kaoru Kobayashi, Shuichi Ohuchida, Yukihiro Sugimoto, Shuh Narumiya, Takashi Sugimura, and Keiji Wakabayashi³

Cancer Prevention Division, National Cancer Center Research Institute, Tokyo 104-0045, Japan [M. M., K. W., T. K., Y. S., M. T., T. K., T. S., K. W.]; Minase Research Institute, Ono Pharmaceutical Co. Ltd., Osaka 618-8585, Japan [K. T., M. K., T. M., K. K., S. O.]; Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto 606-8315, Japan [Y. S.]; and Department of Pharmacology, Faculty of Medicine, Kyoto University, Kyoto 606-8315, Japan [S. N.]

Abstract

Accumulating evidence indicates that overproduction of prostanoids attributable to overexpression of cyclooxygenase-2 (COX-2) plays an important role in colon carcinogenesis. We have shown recently that the prostaglandin (PG) E receptor, EP₁, but not EP₃, is involved in mouse colon carcinogenesis. In line with our previous study, here we examined the role of prostanoid receptors in colon carcinogenesis using six additional lines of knockout mice deficient in prostanoid receptors EP₂, EP₄, DP, FP, IP, or TP. The animals were treated with the colon carcinogen, azoxymethane (AOM), and examined for the development of aberrant crypt foci (ACFs), putative preneoplastic lesions in the colon. Formation of ACFs was decreased only in the EP₄-knockout mice, to 56% of the wild-type level. To confirm these results, we also examined the inhibitory effects of an EP₄-selective antagonist, ONO-AE2-227, in the diet on the formation of AOM-induced colon ACFs in C57BL/6Cr mice and on the development of intestinal polyps in Min mice. ONO-AE2-227 at a dose of 400 ppm reduced the formation of ACFs to 67% of the control level, and intestinal polyp numbers in Min mice receiving 300 ppm were decreased to 69% of the control level. Plating efficiency assays showed that addition of 1.0 μ M ONO-AE1-329, an EP₄-selective agonist, resulted in a 1.8-fold increase in the colony number of the human colon cancer cell line, HCA-7, similar to the effect of PGE₂. Moreover, EP₄ mRNA expression was clearly observed in normal colon mucosa and colon tumors in mice. Our previous and present results indicate that PGE₂ contributes to colon carcinogenesis through its actions mediated through EP₁ and EP₄ receptors; therefore, antagonists for these two receptors may be good candidates as chemopreventive agents against colon cancer.

Introduction

Clear benefits have been reported with NSAIDs⁴ as chemopreventive agents against colon carcinogenesis (1, 2). NSAIDs inhibit arachidonic acid metabolism via actions on COX, a rate-limiting enzyme in the synthesis of PGs, which affect cell proliferation, tumor growth, apoptosis, and immune responsiveness. The presence of two isoforms of COX has been demonstrated—a constitutive enzyme, COX-1, and an inducible enzyme, COX-2—and a number of observations have

suggested that increased activity of this latter plays a critical role in colon carcinogenesis (1–6). Recently, it was reported that genetic disruption of *COX-1*, as well as of *COX-2*, significantly reduces intestinal polyp formation in Min mice having a nonsense mutation in the *Apc* gene (7), so that COX-1 is also suggested to be involved in colon carcinogenesis to some extent.

When considering the possible mechanisms for the chemoprevention of colorectal cancer by NSAIDs, account must be taken of possible PG-independent mechanisms. Studies have shown that NSAIDs cause an increase in cellular arachidonic acid and stimulate the production of sphingomyelinase, resulting in hydrolysis of sphingomyelin to ceramide, which promotes apoptosis of tumor cells (8). Recently, the potential involvement of peroxisome proliferator-activated receptor δ as a adenomatous polyposis coli-regulated target of NSAIDs in colon cancer was demonstrated (9). Moreover, NSAIDs can up-regulate the *prostate apoptosis response 4* gene, a proapoptotic gene, in human colon carcinoma HCA-7 cells (10).

On the other hand, the most striking chemopreventive effects of NSAIDs are thought to be attributable to inhibition of COX with a resultant decrease in PG production. However, it is not fully clear what the legitimate molecular target of PGs is. Prostanoids such as PGE₂, PGD₂, PGF_{2 α} , PGI₂ and thromboxane A₂ exert their biological actions through binding to eight specific membrane receptors; the four subtypes EP₁ to EP₄ for PGE₂; DP for PGD₂; FP for PGF_{2 α} ; IP for PGI₂; and TP for thromboxane A₂ (11, 12). The recent establishment of mice lacking the genes encoding these receptors (13–18) has enhanced our understanding of the involvement of prostanoids and their receptors in the development of colon cancer. In previous studies (19, 20), we demonstrated that PGE₂ contributes to colon carcinogenesis through its binding to the PGE₂ receptor subtype EP₁, using a genetic approach in EP₁-knockout mice and a pharmacological assessment with the EP₁-selective antagonists, ONO-8711 and ONO-8713. The same approach using EP₃-knockout mice indicated that the deficiency of EP₃ receptor has no effect on colon carcinogenesis (19).

The present study was conducted to examine the development of ACFs in six additional lines of mice lacking EP₂, EP₄, DP, FP, IP, or TP. Our results indicate a requirement for the EP₄ receptor in ACF formation by AOM. To confirm these data, we also examined the inhibitory effects of an EP₄-selective antagonist on the formation of colon ACFs induced by AOM in C57BL/6Cr mice and on the development of intestinal polyps in Min mice. Moreover, we determined EP₄ mRNA expression in colonic tissues of mice and examined cell proliferative effects of EP₄ receptor activation using an EP₄-selective agonist. On the basis of the results obtained, the role of EP₄ receptor in colon carcinogenesis is discussed.

Materials and Methods

Animals. Male C57BL/6Cr mice were purchased from Japan SLC, Inc. (Shizuoka, Japan) at 5 weeks of age and female C57BL/6J-Min/+ mice (Min mice) from The Jackson Laboratory (Bar Harbor, ME) at 6 weeks of age. The

Received 9/21/01; accepted 11/15/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported in part by a grant from the Organization for Pharmaceutical Safety and Research of Japan, a Grant-in-Aid for Cancer Research and a Grant-in-Aid for the Second-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labor and Welfare of Japan, and a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, Culture and Technology of Japan.

² These two authors contributed equally to this work.

³ To whom requests for reprints should be addressed, at Cancer Prevention Division, National Cancer Center Research Institute, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan. Phone: 81-3-3542-2511, extension 4500; E-mail: kwakabay@gan2.ncc.go.jp.

⁴ The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; PG, prostaglandin; ACF, aberrant crypt focus; AOM, azoxymethane; ONO-AE2-227, 2-[2-[(2-(1-naphthyl)propanoylamino)phenyl]methyl]benzoic acid; ONO-AE1-329, 16-(3-methoxymethyl)phenyl- ω -tetranor-3,7-dithia-PGE₁; cAMP, cyclic AMP; FBS, fetal bovine serum; RT-PCR, reverse transcription-PCR.

mouse genes encoding each of the six prostanoid receptors, EP₂, EP₄, DP, FP, IP, and TP, were disrupted by a gene knockout method using homologous recombination, as reported previously (13, 15–18). The generated chimeric mice were mated with C57BL/6Cr mice to produce heterozygotes for the respective alleles. The heterozygotes were backcrossed with C57BL/6Cr mice to exclude possible effects of genetic background. The resulting heterozygous male mice were intercrossed, and the F₂ progeny of the wild-type and homozygous mutant mice were used at 9 (EP₂, FP, IP, and TP) or 13 (DP) weeks of age. In the case of the EP₄-knockout mice, chimeric mice were mated to C57BL/6Cr mice, and homozygous mutants were obtained by interbreeding of the resulting agouti offspring. Most EP₄-deficient neonates with 129 × C57BL background become lethargic within 72 h after birth because of a patent ductus arteriosus, and <5% survive and grow normally (16). We used male EP₄-knockout mice that survived for analysis of ACF formation starting at 7 weeks of age. Genotypes of the knockout mice were confirmed by PCR according to the method described previously (13, 15–18). The animals were housed in plastic cages at 24 ± 2°C and 55% relative humidity with a 12/12-h light/dark cycle. Water and basal diet (AIN-76A; Bio-Serv, Frenchtown, NJ) or experimental diets, with addition of an EP₄-selective antagonist at the indicated concentrations with thorough mixing, prepared every week, were given *ad libitum*. Body weights and food intake were measured weekly. The experimental protocol was approved by the Institutional Ethics Review Committee for animal experimentation.

AOM-induced ACF Formation in Prostanoid Receptor-Knockout Mice. EP₂, EP₄, DP, FP, IP, and TP-knockout [EP₂^{-/-} (male, *n* = 7), EP₄^{-/-} (male, *n* = 10), DP^{-/-} (female, *n* = 11), FP^{-/-} (male, *n* = 9), IP^{-/-} (male, *n* = 10), and TP^{-/-} (male, *n* = 11)] mice and counterpart wild-type mice (*n* = 7–11/group) were treated with AOM (Sigma Chemical Co., St. Louis, MO) at a dose of 10 mg/kg body weight i.p. once a week for 3 weeks. All mice were sacrificed 5 weeks after the first dose of AOM. After laparotomy, the entire colons were resected and filled with 10% neutral buffered formalin and then opened longitudinally from the anus to the cecum. Each was fixed flat between sheets of filter paper in 10% neutral buffered formalin and then stained with 0.2% methylene blue in saline and scored under a light microscope for the number of ACFs/colon, number of ACFs/colon, and mean number of ACFs/focus according to the procedure of Bird (21).

The Selective EP₄ Antagonist, ONO-AE2-227. The selective EP₄ receptor antagonist, ONO-AE2-227, was chemically synthesized at Ono Pharmaceutical Co., Ltd. Receptor binding experiments with this compound were conducted using Chinese hamster ovary cell lines, stably expressing each type of mouse prostanoid receptor. The *K_i* values were found to be 2.7 nM for the mouse EP₄ receptor and 21 nM for mouse EP₃ receptor. The *K_i* values for the other receptors, mouse EP₁, EP₂, DP, FP, IP, and TP receptors were >1000 times higher than that for the mouse EP₄ receptor. Analysis of its agonistic and antagonistic actions showed the compound to act as a potent and competitive antagonist to the EP₄ receptor; it inhibited PGE₂ (100 nM)-induced increase in cytosolic cAMP concentration with a median inhibitory concentration of 10 nM. ONO-AE2-227 also acted as a relatively weak antagonist to the EP₃ receptor; it inhibited the PGE₂ (10 nM)-induced increase in cytosolic calcium concentration with an IC₅₀ of 160 nM. Details for the chemical synthesis and biological activities of ONO-AE2-227 will be reported elsewhere. By high performance liquid chromatography, ONO-AE2-227 was confirmed to be stable for at least 4 weeks at ambient temperature in the diet.

Effects of ONO-AE2-227 on Formation of AOM-induced ACF in C57BL/6Cr Mice and Intestinal Polyps in Min Mice. C57BL/6Cr male mice, 6 weeks of age, were given i.p. injections of AOM or the vehicle, as described in the experiments for the different lines of prostanoid receptor-knockout mice. The mice in the EP₄-selective antagonist-treated groups were fed diets containing 100 or 400 ppm of ONO-AE2-227 starting the day before the first AOM dosing until the end of the experiment at week 5. Numbers of AOM-injected mice were eight for each group, and those for vehicle-injected mice were three for the 400 ppm of experimental diet groups and three for the control diet group. ACF in the colon of mice were assessed as described above.

Groups of 10 female Min mice were fed diet containing ONO-AE2-227 or basal diet from 6 weeks of age until the termination of the experiment 7 weeks thereafter. It is expected that C57BL/6Cr mice are generally much resistant to chemical treatment than Min mice. In addition, the experimental period with Min mice was longer than that of ACF induction in the experiment with C57BL/6Cr. Therefore, the dose of 300 ppm of ONO-AE2-227 was chosen for

the experiment. After sacrifice and laparotomy, the entire intestinal tract was resected, filled with 10% neutral buffered formalin, and divided into four sections: the colon and three sections of the small intestine, including the proximal (~4 cm from the pylorus ring of stomach), middle (the proximal half of the remainder), and distal parts. These sections were opened longitudinally and fixed flat between sheets of filter paper in 10% neutral buffered formalin, and the numbers and sizes of polyps were determined under a stereoscopic microscope.

Effects of ONO-AE1-329 and PGE₂ on Colony Formation of HCA-7 Cells. HCA-7 colony 29, a human colon adenocarcinoma cell line, was kindly provided from Dr. Susan Kirkland (Imperial College of Science, Technology and Medicine, London, United Kingdom). The cells were maintained in DMEM supplemented with 10% heat-inactivated FBS (Hyclone Laboratories, Inc., Logan, UT) and antibiotics (100 µg/ml of streptomycin and 100 units/ml of penicillin) at 37°C in 5% CO₂. The numbers of HCA-7 cell colonies were counted as described previously (22) with slight modifications. In brief, HCA-7 cells were plated in 6-cm cell culture dishes at a density of 1000 cells/dish, with DMEM containing 10% FBS. The selective EP₄ receptor agonist, ONO-AE1-329 (23), or PGE₂ (Cayman Chemical Co., MI) was added daily to selected cells, and the medium was also replaced every day. Cells were incubated for 14 days, and then colonies were visualized by staining with 0.2% methylene blue and counted manually.

Analysis of EP₄ Expression by RT-PCR in Colon Cancer Samples from AOM-treated Mice. Male C57BL/6J mice (CLEA Japan, Inc., Tokyo, Japan) at 7 weeks of age were i.p. injected with 10 mg/kg body weight of AOM once a week for a total of six times to obtain many colon tumors, as described previously (3), and sacrificed at 50 weeks after the first injection. Their colons were removed, and one half of each of five colon tumors and five neighboring normal mucosa samples were immediately frozen, stored at -80°C, and used for RT-PCR analyses. The samples were sonicated, and total RNAs were isolated using ISOGEN (Nippon Gene Co., Tokyo, Japan). One-µg aliquots of total RNA were subjected to the reverse transcription reaction using an RNA LA PCR kit (Takara Shuzo Co., Shiga, Japan). Oligonucleotide primers specific for mouse EP₄ (5'-TTCCGCTCGTGGTGCGAGTGTTC-3'; 5'-GAGGTGGTGTCTGCTTGGGTCAG-3') were used for amplification of each mRNA. All PCR reactions were performed in a final volume of 50 µl for 30 cycles. The PCR products were analyzed by 2% agarose gel electrophoresis.

Statistical Analysis. The data for ACF and polyp formation are expressed as mean ± SE, and their statistical analysis was carried out with the Student's *t* test. Differences were considered statistically significant at *P* < 0.05.

Results

ACF Development in Prostanoid Receptor-Knockout Mice. To determine which prostanoid receptors might be involved in colon carcinogenesis, we used a short-term *in vivo* model using ACF formation induced by AOM as the end point. The mean body weights of the AOM-treated EP₄-knockout mice were comparable with those of AOM-treated wild-type mice. No abnormal clinical signs were observed during the course of the experiment, and no change was evident in organ weights including the liver, kidneys, and spleen between the two groups. ACFs were noted in all animals treated with AOM and were located mainly in the distal colon, with fewer numbers in the middle colon and rectum. In wild-type (EP₄^{+/+}, *n* = 10) and knockout (EP₄^{-/-}, *n* = 10) mice, the numbers of ACFs/colon were 14.6 ± 2.0 and 8.2 ± 1.4 (*P* < 0.05), and the mean numbers of ACFs/focus were 1.5 ± 0.1 and 1.5 ± 0.1, respectively. Thus, the numbers of ACFs per colon in EP₄-knockout mice were significantly reduced to 56% of the wild-type value. Mice treated with saline showed no evidence of ACF formation in either knockout or wild-type mice.

Under the same conditions, the effects of deficiency of EP₂, DP, FP, IP, or TP receptors on formation of ACFs were examined. As with the EP₄-knockout mice, no abnormal changes in body or organ weights were observed in knockout mice compared with wild-type mice, except for a slight increase in spleen weights of IP-knockout mice. There were no significant differences in the numbers of ACFs/

colon in EP₂, DP, FP, IP, and TP-knockout mice from those of their wild-type counterparts. Moreover, the mean numbers of ACFs/focus in these receptor-knockout mice groups did not differ from those in the wild-type mice. Fig. 1 summarizes the data for the effects of six prostanoind receptor deficiencies on AOM-induced ACF in mice. For reference, the results for EP₁- and EP₃-knockout mice, reported previously (19), are also included in Fig. 1.

Suppression of AOM-induced ACF Formation by the EP₄-selective Antagonist in C57BL/6Cr Mice. To confirm a role for the EP₄ receptor in colon carcinogenesis, we investigated the effects of ONO-AE2-227, a selective EP₄ antagonist, on the formation of ACF induced by AOM in C57BL/6Cr mice. Administration of diet containing 100 or 400 ppm of ONO-AE2-227 did not affect the body and organ weights in the AOM-injected groups. ACFs were observed in all animals ($n = 8$ for each group) treated with AOM. Administration of 400 ppm of ONO-AE2-227 to AOM-treated mice throughout the experiment for 5 weeks significantly decreased the numbers of ACFs/colon (8.3 ± 1.1 , $P < 0.05$) to 67% of that (12.4 ± 2.0) for the AOM-alone group. The mean numbers of ACFs/focus in the two groups were both 1.5 ± 0.1 . The numbers of ACFs/colon and the mean numbers of ACFs/focus in 100 ppm of the ONO-AE2-227 group were 12.4 ± 1.6 and 1.5 ± 0.1 , respectively. Thus, administration of 100 ppm of ONO-AE2-227 did not affect ACF formation. No ACFs were observed in vehicle-injected mice, with or without 400 ppm ONO-AE2-227.

Suppression of Intestinal Polyp Formation by the EP₄-selective Antagonist in Min Mice. Administration of ONO-AE2-227 at a dose of 300 ppm in the diet for 7 weeks did not affect the body weights, feeding, or behavior of Min mice. Data for number and distribution of intestinal polyps in the basal diet and ONO-AE2-227 groups are shown in Table 1. Most polyps were located in the small intestine with only a few in the colon. Administration of ONO-AE2-227 significantly reduced the total number of polyps to 69% of that in the basal diet group. The number of polyps detected in the distal portion of the small intestines was significantly lower (65% of the basal diet group value), and that in the middle portion was also lower (74% of the basal diet group value), although this was not statistically significant. Fig. 2 shows the size distributions of intestinal polyps in the basal diet and ONO-AE2-227-treated groups. Treatment with the EP₄-selective antagonist significantly reduced the number of polyps

Table 1 Suppression of intestinal polyp development by ONO-AE2-227 in Min mice

Mice were fed basal diet or diet containing 300 ppm of ONO-AE2-227 for 7 weeks. Numbers of Min mice fed the experimental and control diets were ten each. Data are mean \pm SE. Numbers in parentheses indicate percentage compared with the basal diet group.

Polyp location	No. of polyps/mouse	
	Basal diet	ONO-AE2-227
Proximal small intestine	4.0 ± 0.4	3.3 ± 1.1 (83)
Middle small intestine	19.3 ± 2.0	14.2 ± 2.2 (74)
Distal small intestine	37.6 ± 5.3	24.5 ± 2.0^a (65)
Colon	0.5 ± 0.2	0.2 ± 0.1 (40)
Total	61.4 ± 7.3	42.2 ± 4.3^a (69)

^a $P < 0.05$ versus the basal diet group.

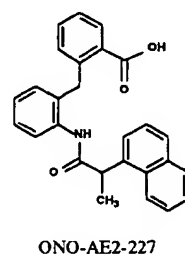
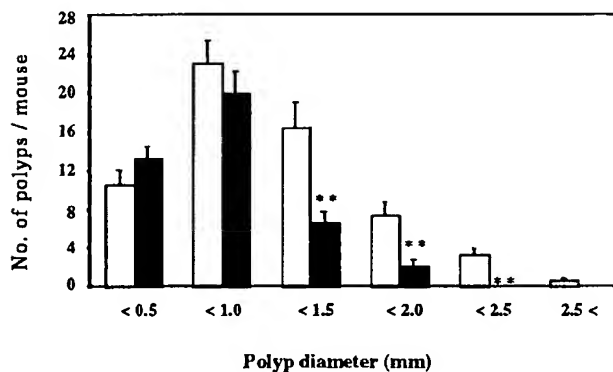


Fig. 2. Size distribution of intestinal polyps in Min mice. □, basal diet; ■, 300 ppm ONO-AE2-227. (The structure is shown in lower part of the figure). Polyps were classified in terms of their diameters in millimeters. The number of polyps/mouse in each size class is expressed as the means; bars, SE. **, $P < 0.01$, compared with the basal diet group.

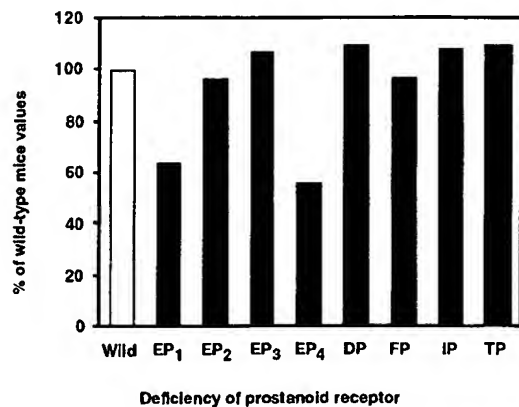


Fig. 1. Numbers of ACFs developing in the colons of prostanoind receptor-knockout mice and their wild-type counterparts after treatment with AOM. EP₁, EP₂, EP₃, EP₄, DP, FP, IP, and TP-knockout mice and their respective wild-type mice were treated with AOM, and the numbers of ACFs were scored at 5 weeks after the first dose of AOM. The values (numbers of ACFs/colon) for each knockout mouse group are expressed as percentages of those for their wild-type counterparts. The data for EP₁- and EP₃-knockout mice are from our previously published work (19). □, wild-type mice; ■, knockout mice. *, $P < 0.05$, compared with the wild-type mice.

measuring ≥ 1.0 mm in diameter but not those measuring < 1.0 mm in diameter.

Effects of the EP₄-Selective Agonist and PGE₂ Treatment on Colony Formation of HCA-7 Cells. To evaluate the physiological functions of the EP₄ receptor, we examined the effects of the EP₄ receptor-selective agonist, ONO-AE1-329, and PGE₂ treatment on colony formation in monolayer cultures. We used a human colon epithelial cell line, HCA-7 cells, in which expression of the EP₄ receptor could be detected by RT-PCR analysis (data not shown). For this experiment, 1000 cells were seeded in six-cm dishes, and ONO-AE1-329 was added daily at concentrations of 0.1, 1.0, and 10 μ M in fresh medium for 14 days. We observed a 1.8-fold increase in HCA-7 colony number in the presence of 1.0 μ M ONO-AE1-329 and a 1.5-fold increase in the presence of 1.0 μ M PGE₂ for 14 days (Fig. 3). The highest dose of ONO-AE1-329 (10 μ M) decreased the HCA-7 colony number.

EP₄ mRNA Expression in Normal Colon and Colon Cancer Tissues in Mice. The expression of EP₄ mRNA in normal colon mucosal and tumor tissues from five mice was examined. Representative data are shown in Fig. 4. EP₄ mRNA was detected in all colon tumor and normal mucosa samples by RT-PCR. All of the tumors

sampled were histopathologically diagnosed as well-differentiated adenocarcinomas.

Discussion

In the present study, examination of the effects of EP₂, EP₄, DP, FP, IP, and TP receptor knockout on AOM-induced ACF formation in mice provided evidence for the involvement of the PGE₂ receptor subtype EP₄ but not EP₂, DP, FP, IP, or TP in colon carcinogenesis. In addition, administration of an EP₄-selective antagonist, ONO-AE2-227, to AOM-treated C57BL/6Cr mice and Min mice decreased ACFs and intestinal polyp formation, respectively. Interestingly, in the latter case the number of polyps ≥ 1.0 mm in diameter, but not those < 1.0 mm in diameter, were reduced, suggesting reduction in tumor growth. An EP₄-selective agonist, ONO-AE1-329, was further found to increase colony formation by HCA-7 cells, similar to PGE₂. Moreover, we could demonstrate the expression of EP₄ receptors in colon tumors and normal mucosa, in line with earlier results of *in situ* hybridization for EP₄ mRNA (24). In the previous study, EP₁ receptor was shown to be involved in colon carcinogenesis (19). In addition, our preliminary study indicated that EP₁ receptor expression was detected in AOM-induced colon tumors in mice by RT-PCR analysis (data not shown). Thus, combined together, our present and previous results suggest that PGE₂ mediates carcinogenic changes by acting at EP₁ and EP₄ receptors in the colon. Consistent with these data, increased PGE₂ levels in colon tumor tissues compared with the surrounding normal mucosa were suggested to play an important role in colon carcinogenesis (25).

PGE₂ was earlier suggested to stimulate an increase in cell proliferation and motility of the colon cancer cell line LS-174 by activating the phosphatidylinositol 3-kinase/Akt pathway via EP₄ receptor activation (26). It is also known that PGE₂ activates adenylate cyclase via a cholera toxin-sensitive, stimulatory G protein through binding to the EP₄ receptor. In the adenylate cyclase pathway, increased cAMP levels result in an activation of cAMP-dependent protein kinase (PKA) and a transcriptional factor that binds to cAMP-responsive elements to transactivate the transcription of specific primary response genes that initiate cell proliferation (27). These biological changes could contribute to colon carcinogenesis through EP₄ receptor involvement. The EP₁ receptor is a transmembrane G protein-coupled receptor, similar to other PGE₂ receptors, but its signal transduction mechanism is not known in detail. EP₁ signals are transmitted by increased intracellular Ca²⁺ concentrations and activate protein kinase C (11, 12). Additional studies are needed to

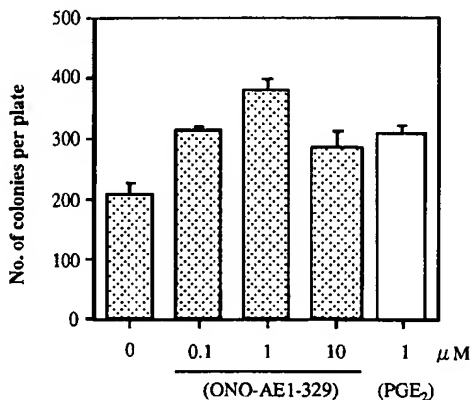


Fig. 3. HCA-7 colony number in response to treatment with ONO-AE1-329 or PGE₂. HCA-7 cells were plated in six-cm cell culture dishes at a density of 1000 cells/dish, with DMEM containing 10% FBS. Cells were incubated for 14 days, and then colonies were visualized by staining with 0.2% methylene blue and counted manually. Data are means ($n = 3$); bars, SE. Similar results were obtained in three separate experiments.



Fig. 4. EP₄ receptor expression in normal colon and colon cancer tissues of mice. Aliquots of total RNA (1 μ g) from colon cancers and neighboring normal colon tissues were subjected to RT-PCR. The reaction mixture for mouse EP₄ was first heated at 94°C for 2 min, followed by amplification for 30 cycles at 95°C for 30 s, 60°C for 45 s, and 72°C for 4 min. These temperatures and time intervals were followed by a final incubation at 72°C for 5 min. The PCR products were separated on 2% agarose gels, and portions corresponding to the expected DNA size (488 bp) of the PCR products are shown in the middle of the figure with an arrow. N, normal mucosa; T, tumor.

investigate events downstream of the EP₁ receptor signaling pathway and any link between EP₁ and EP₄ receptors. Recently, it was reported that homozygous deletion of the gene encoding EP₂ receptor resulted in decrease of intestinal polyp formation in the *Apc* knockout mice (28). These data are not consistent with the results obtained in the present study. Therefore, involvement of EP₂ receptor in AOM-induced colon carcinogenesis in rodents and intestinal polyp formation in Min mice needs to be examined using EP₂ receptor antagonists.

Selective inhibitors of COX-2 are good candidates as chemopreventive agents, with clinically important mechanism-based safety characteristics that significantly distinguish them from traditional NSAIDs, which suffer from gastrointestinal side effects that limit long-term application. It might be expected that these adverse effects are further diminished by inhibiting the downstream of COX pathway. On the basis of the present results, selective EP₁ and/or EP₄ receptor antagonists may be particularly beneficial as chemopreventive agents for colon cancer with low toxicity.

In conclusion, the data obtained in our present and previous studies suggest that PGE₂ mediates colonic carcinogenic changes by acting at EP₁ and EP₄ receptors in the colon. For confirmation, long-term colon carcinogenesis experiments with EP₁ and EP₄ antagonists are currently being conducted in our laboratory.

References

- Smalley, W. E., and DuBois, R. N. Colorectal cancer and nonsteroidal anti-inflammatory drugs. *Adv. Pharmacol.*, 39: 1-20, 1997.
- Elder, D. J. E., and Paraskeva, C. COX-2 inhibitors for colorectal cancer. *Nat. Med.*, 4: 392-393, 1998.
- Fukutake, M., Nakatsugi, S., Itoi, T., Takahashi, M., Ohta, T., Mamiya, S., Taniguchi, Y., Sato, H., Fukuda, K., Sugimura, T., and Wakabayashi, K. Suppressive effects of nimesulide, a selective inhibitor of cyclooxygenase-2, on azoxymethane-induced colon carcinogenesis in mice. *Carcinogenesis (Lond.)*, 19: 1939-1942, 1998.
- Kawamori, T., Rao, C. V., Seibert, K., and Reddy, B. S. Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. *Cancer Res.*, 58: 409-412, 1998.
- Steinbach, G., Lynch, P. M., Phillips, R. K., Wallace, M. H., Hawk, E., Gordon, G. B., Wakabayashi, N., Saunders, B., Shen, Y., Fujimura, T., Su, L. K., and Levin, B. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *N. Engl. J. Med.*, 342: 1946-1952, 2000.
- Oshima, M., Dinchuk, J. E., Kargman, S. L., Oshima, H., Hancock, B., Kwong, E., Trzaskos, J. M., Evans, J. F., and Taketo, M. M. Suppression of intestinal polyposis in *Apc*^{Δ716} knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell*, 87: 803-809, 1996.
- Chulada, P. C., Thompson, M. B., Mahler, J. F., Doyle, C. M., Gaul, B. W., Lee, C., Tiano, H. F., Morham, S. G., Smithies, O., and Langenbach, R. Genetic disruption of *Ptgs-1*, as well as *Ptgs-2*, reduces intestinal tumorigenesis in Min mice. *Cancer Res.*, 60: 4705-4708, 2000.
- Chan, T. A., Morin, P. J., Vogelstein, B., and Kinzler, K. W. Mechanisms underlying nonsteroidal antiinflammatory drug-mediated apoptosis. *Proc. Natl. Acad. Sci. USA*, 95: 681-686, 1998.
- He, T. C., Chan, T. A., Vogelstein, B., and Kinzler, K. W. PPAR δ is an APC-regulated target of nonsteroidal anti-inflammatory drugs. *Cell*, 99: 335-345, 1999.

10. Zhang, Z., and DuBois, R. N. *Par-4*, a proapoptotic gene, is regulated by NSAIDs in human colon carcinoma cells. *Gastroenterology*, 118: 1012–1017, 2000.
11. Coleman, R. A., Smith, W. L., and Narumiya, S. International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacol. Rev.*, 46: 205–229, 1994.
12. Ushikubi, F., Hirata, M., and Narumiya, S. Molecular biology of prostanoid receptors: an overview. *J. Lipid Mediat. Cell Signalling*, 12: 343–359, 1995.
13. Matsuoka, T., Hirata, M., Tanaka, H., Takahashi, Y., Murata, T., Kabashima, K., Sugimoto, Y., Kobayashi, T., Ushikubi, F., Aze, Y., Eguchi, N., Urade, Y., Yoshida, N., Kimura, K., Mizoguchi, A., Honda, Y., Nagai, H., and Narumiya, S. Prostaglandin D₂ as a mediator of allergic asthma. *Science (Wash. DC)*, 287: 2013–2017, 2000.
14. Ushikubi, F., Segi, E., Sugimoto, Y., Murata, T., Matsuoka, T., Kobayashi, T., Hizaki, H., Tsuboi, K., Katsuyama, M., Ichikawa, A., Tanaka, T., Yoshida, N., and Narumiya, S. Impaired febrile response in mice lacking the prostaglandin E receptor subtype EP₃. *Nature (Lond.)*, 395: 281–284, 1998.
15. Hizaki, H., Segi, E., Sugimoto, Y., Hirose, M., Saji, T., Ushikubi, F., Matsuoka, T., Noda, Y., Tanaka, T., Yoshida, N., Narumiya, S., and Ichikawa, A. Abortive expansion of the cumulus and impaired fertility in mice lacking the prostaglandin E receptor subtype EP₂. *Proc. Natl. Acad. Sci. USA*, 96: 10501–10506, 1999.
16. Segi, E., Sugimoto, Y., Yamasaki, A., Aze, Y., Oida, H., Nishimura, T., Murata, T., Matsuoka, T., Ushikubi, F., Hirose, M., Tanaka, T., Yoshida, N., Narumiya, S., and Ichikawa, A. Patent ductus arteriosus and neonatal death in prostaglandin receptor EP₄-deficient mice. *Biochem. Biophys. Res. Commun.*, 246: 7–12, 1998.
17. Sugimoto, Y., Yamasaki, A., Segi, E., Tsuboi, K., Aze, Y., Nishimura, T., Oida, H., Yoshida, N., Tanaka, T., Katsuyama, M., Hasumoto, K., Murata, T., Hirata, M., Ushikubi, F., Negishi, M., Ichikawa, A., and Narumiya, S. Failure of parturition in mice lacking the prostaglandin F receptor. *Science (Wash. DC)*, 277: 681–683, 1997.
18. Murata, T., Ushikubi, F., Matsuoka, T., Hirata, M., Yamasaki, A., Sugimoto, Y., Ichikawa, A., Aze, Y., Tanaka, T., Yoshida, N., Ueno, A., Oh-ishi, S., and Narumiya, S. Altered pain perception and inflammatory response in mice lacking prostacyclin receptor. *Nature (Lond.)*, 388: 678–682, 1997.
19. Watanabe, K., Kawamori, T., Nakatsugi, S., Ohta, T., Ohuchida, S., Yamamoto, H., Maruyama, T., Kondo, K., Ushikubi, F., Narumiya, S., Sugimura, T., and Wakabayashi, K. Role of the prostaglandin E receptor subtype EP₁ in colon carcinogenesis. *Cancer Res.*, 59: 5093–5096, 1999.
20. Watanabe, K., Kawamori, T., Nakatsugi, S., Ohta, T., Ohuchida, S., Yamamoto, H., Maruyama, T., Kondo, K., Narumiya, S., Sugimura, T., and Wakabayashi, K. Inhibitory effect of a prostaglandin E receptor subtype EP₁ selective antagonist, ONO-8713, on development of azoxymethane-induced aberrant crypt foci in mice. *Cancer Lett.*, 156: 57–61, 2000.
21. Bird, R. P. Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. *Cancer Lett.*, 37: 147–151, 1987.
22. Sheng, H., Shao, J., Morrow, J. D., Beauchamp, R. D., and DuBois, R. N. Modulation of apoptosis and Bcl-2 expression by prostaglandin E₂ in human colon cancer cells. *Cancer Res.*, 58: 362–366, 1998.
23. Yamane, H., Sugimoto, Y., Tanaka, S., and Ichikawa, A. Prostaglandin E₂ receptors, EP₂ and EP₄, differentially modulate TNF- α and IL-6 production induced by lipopolysaccharide in mouse peritoneal neutrophils. *Biochem. Biophys. Res. Commun.*, 278: 224–228, 2000.
24. Morimoto, K., Sugimoto, Y., Katsuyama, M., Oida, H., Tsuboi, K., Kishi, K., Kinoshita, Y., Negishi, M., Chiba, T., Narumiya, S., and Ichikawa, A. Cellular localization of mRNAs for prostaglandin E receptor subtypes in the mouse gastrointestinal tract. *Am. J. Physiol.*, 272: G681–G687, 1997.
25. Rigas, B., Goldman, I. S., and Levine, L. Altered eicosanoid levels in human colon cancer. *J. Lab. Clin. Med.*, 122: 518–523, 1993.
26. Sheng, H., Shao, J., Washington, M. K., and DuBois, R. N. Prostaglandin E₂ increases growth and motility of colorectal carcinoma cells. *J. Biol. Chem.*, 276: 18075–18081, 2001.
27. Dhanasekaran, N., Tsim, S. T., Dermott, J. M., and Onesime, D. Regulation of cell proliferation by G proteins. *Oncogene*, 17: 1383–1394, 1998.
28. Sonoshita, M., Takaku, K., Sasaki, N., Sugimoto, Y., Ushikubi, F., Narumiya, S., Oshima, M., and Taketo, M. M. Acceleration of intestinal polyposis through prostaglandin receptor EP₂ in *Apc* ^{Δ 716} knockout mice. *Nat. Med.*, 7: 1048–1051, 2001.